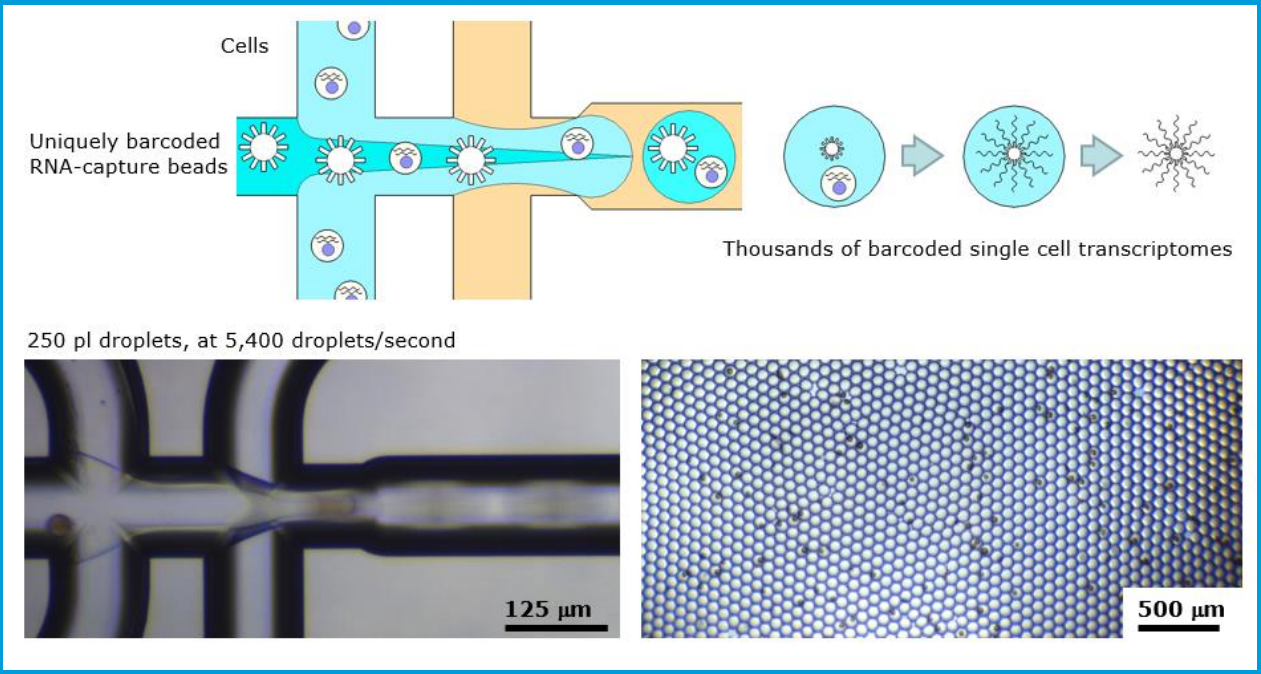
RNA-Seq application note

Encapsulating single beads with cells on the RNA-Seq chip



dolomite

Summary

Many biologically important processes take place at the level of single cells, including processes in neurobiology, development, immune responses and cancer. However, many traditional techniques, such as Western blots and PCR involve homogenising tissue samples, so can only deal with population averages, and much crucial information is lost. Droplet microfluidics offers the unique ability to isolate thousands to millions of cells in individual droplets. A very exciting method for high throughput single cell RNA-Seq has been published recently (Macosko E., et al. "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets." *Cell* **161**:1202). In the Drop-seq method, tens of thousands of cells are individually encapsulated with uniquely barcoded RNA capture beads, leading to the robust and straightforward preparation of tens of thousands of single cell cDNA libraries. In this application note, we demonstrate the single cell RNA-Seq method on a Dolomite droplet system.

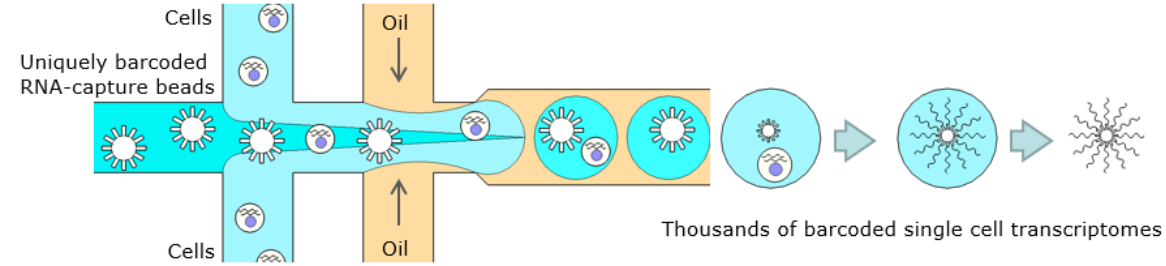


Figure 1. **Schematic of single cell RNA-Seq method**. Tens of thousands of single cells are encapsulated with uniquely barcoded mRNA-capture beads. The cells are lysed in the droplets, and beads capture mRNA from single cells. The single cell RNA-beads are recovered and processed for downstream high throughput sequencing.

Introduction

Cell encapsulation in microfluidic droplets is an exciting, cutting edge technique that, for the first time, enables expression analysis of thousands or even millions of single cells. This unprecedented analytical power allows the exhaustive discovery of previously unidentified or cryptic cell types in the tissue of interest, and also allows their behaviour to be followed through biologically important processes. Because the volume of the droplets is small – in the range of nanolitres to picolitres – mRNA capture or reverse transcription from single cells is efficient and reliable, and the technique is within the reach of most well-staffed research labs.

A pair of very exciting methods for high throughput single cell RNA-Seq has been published recently (Macosko E., et al. "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets." *Cell* **161**:1202; Klein, AM., et al. "Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic Stem Cells." *Cell* **161**:1187). The Drop-seq protocol involves encapsulating single cells with single barcoded beads. The barcoded oligo bead library is constructed such that each bead has a unique DNA barcode sequence, but within a bead, the thousands of copies of oligo all

contain an identical barcode sequence. The 3' end of the oligo has a poly(dT) stretch that captures mRNA and primes reverse transcription.

It is desired to encapsulate single cells with single barcoded beads, and this is achieved by limiting dilution. In general, there is a trade-off between cleaner data (more dilute cells and beads) and higher throughput, or more efficient capture of cells (more concentrated cells and/or beads). The dilution of cells or beads can be chosen based on the desired outcome of the experiment. If there are two cells in a droplet with a bead, then the bead will capture mRNA from two cells. In a discovery experiment, this could be spuriously interpreted as a new cell type. If the cell types are already known, it can be identified and discarded computationally. If there are two beads in a droplet with one cell, this is typically less confounding, because it just means the cell is identified or counted twice. As long as the cell identify is independent of the probability of being encapsulated with two beads (which it almost always will be), then it won't affect the statistics.

For cleaner data, a researcher may choose a dilution where 1 in 20 droplets contains a cell or bead. In this case, it means that 1 in 400 droplets contains a cell and a bead (1/20 X 1/20). This also means that only 1 in 20 cells will be encapsulated with a bead, and the rest will effectively be discarded. If a researcher desires to recover data from most of the cells, it may make sense to encapsulate cells at 1 cell per 20 droplets, but increase the bead concentration, so that more droplets contain beads, and more of the cells have their mRNA captured on beads. In this case, the droplets that contain one cell and two beads do not cause a problem.

Cells are typically encapsulated in aqueous droplets in fluorocarbon oils such as FC40 or HFE 7500. Fluorocarbon oils are biocompatible and allow gas exchange (some have been tested for total liquid ventilation or as blood substitutes).

Cell encapsulation is fast becoming a mature technology and now is rapidly transitioning from an engineering demonstration to a research tool being used by skilled researchers in many advanced biological laboratories. The original cell encapsulation microfluidic systems used in the initial research were rather cutting edge, bespoke experimental systems built by engineers within research laboratories. These prototype systems are not necessarily easy for biologists to create or use, or suited to use in a day-to-day research work-flow.

Dolomite is a world-leader in the design and manufacture of microfluidic systems and components. Dolomite specialises in 'Productising Science®', which aims to provide microfluidic solutions for research scientists. Dolomite has complete cell encapsulation systems which are modular, and include components such as high pressure pumps and sensors, highly reliable glass microfluidic chips, easy-to-use microfluidic connectors, and automation software.

This application note reports some preliminary results of single cell RNA sequencing using the Dolomite cell encapsulation system.

Materials and methods

Droplet system. The cell encapsulation system uses pulseless Dolomite P-Pumps, and can be driven from a PC via the Flow Control Centre software. The beads and cells were loaded into sample loops, which allow the beads and cells to be flowed without mechanical stirring to avoid fragmenting the beads, and premature lysis of the cells. The upstream end of the sample loops have 4-way sample injection valves. A two way valve ('shut-off' valve) was included on the oil line to prevent backflow on shutting the system down. The P-Pumps were controlled from the Dolomite Flow Control Centre software, in flow control mode.

High speed microscope. The Dolomite high speed microscope is a simple compact microscope, with a convenient long working distance lens. There are no eye-pieces, partly to protect users from the very bright light source. The microscope allows short exposure times (50 μs), which is useful in monitoring high speed droplet production.

Glass Drop-seq chip. A prototype PDMS Drop-seq chip was described in the literature (Macosko et al.). For standard products, glass is preferred, as the glass chips are robust, highly reproducible, and chemically inert. We therefore produced a glass Drop-seq chip. The channels allow the use of robust, standard, leak-free connectors, while allowing the junction to be readily imaged. The chips have two identical microfluidic circuits per chip, and were fluorophilically coated.

Advantageously, the glass Drop-seq chip has a higher throughput than the PDMS chip. Specifically, the glass Drop-seq chip makes 330 pl droplets at 4 kHz (4,000 droplets/second), at flow rates of 40 μl/min each for the bead and cell suspensions, and 200 μl/minute for oil. This higher throughput allows samples to be processed more rapidly. The only adjustments required to the published Drop-seq protocol are to suspend the cells and beads at a higher concentration, and to use the slightly lower flow rates described in this application note.

Beads. The beads described in the protocol (~30 μm) were suspended in 7% Ficoll, 0.2% Sarkosyl and 1X TBS. A 1:1 slurry of beads in water was prepared, and approximately 25 μl of this slurry was added per 1 ml of lysis buffer. The bead concentration was counted in a hemacytometer. Following a test encapsulation, the droplets and beads were counted, and the necessary adjustment to the concentration to obtain 1 bead per 20 droplets was calculated.

Flowing beads. As noted in Macosko et al., the oligo beads sediment out of suspension rapidly, so a magnetic mixing disc was included in the syringes to stir the beads, which resulted in some bead fragmentation. To avoid this fragmentation, a number of methods

Table 1. Droplet sizes, and single cell library formation rates

droplet diameter, μm	75	85	100	125
droplet volume, pl	221	322	524	1,023
droplets/second, at 2X 40 μl/min aqueous flows	6,036	4,147	2,546	1,304
cells/ml, for 1 cell/20 droplets	4.5×10^5	3.1×10^5	1.9×10^4	9.8×10^3
Single cell libraries/minute, at 1 cell/20 droplets	905	622	382	196

of flowing beads and cells were investigated, namely using ‘rotating’ a microcentrifuge tube in the P-Pump reservoir, a sample loop, and a ‘rocked’ sample loop.

‘Rotating’ the microcentrifuge tube containing the sample. 500 μl of beads and 500 μl of cells were placed in microcentrifuge tubes, which were placed in the chamber of respective remote P-Pumps, and the chambers placed on a magnetic stirrer. A 20 mm magnetic stir bar was also placed in the chamber, outside of the microcentrifuge tube, such that when the stir bar rotated (120 – 180 rpm), the microcentrifuge tube also rotated, keeping the beads in suspension.

Sample loops. Sample loops were used to allow pumping of the beads and cells without mechanical agitation and the consequent risk of mechanical shearing (leading to broken beads (which can then end up in more than one droplet), or prematurely lysed cells. Sample loops were 10m of 0.24 mm ID tubing, with a volume of 0.5 ml, and were connected to the upstream sample injection valve with a nut and ferrule. Samples were injected into the loop with a 1 ml syringe. The sample was first aspirated into the syringe

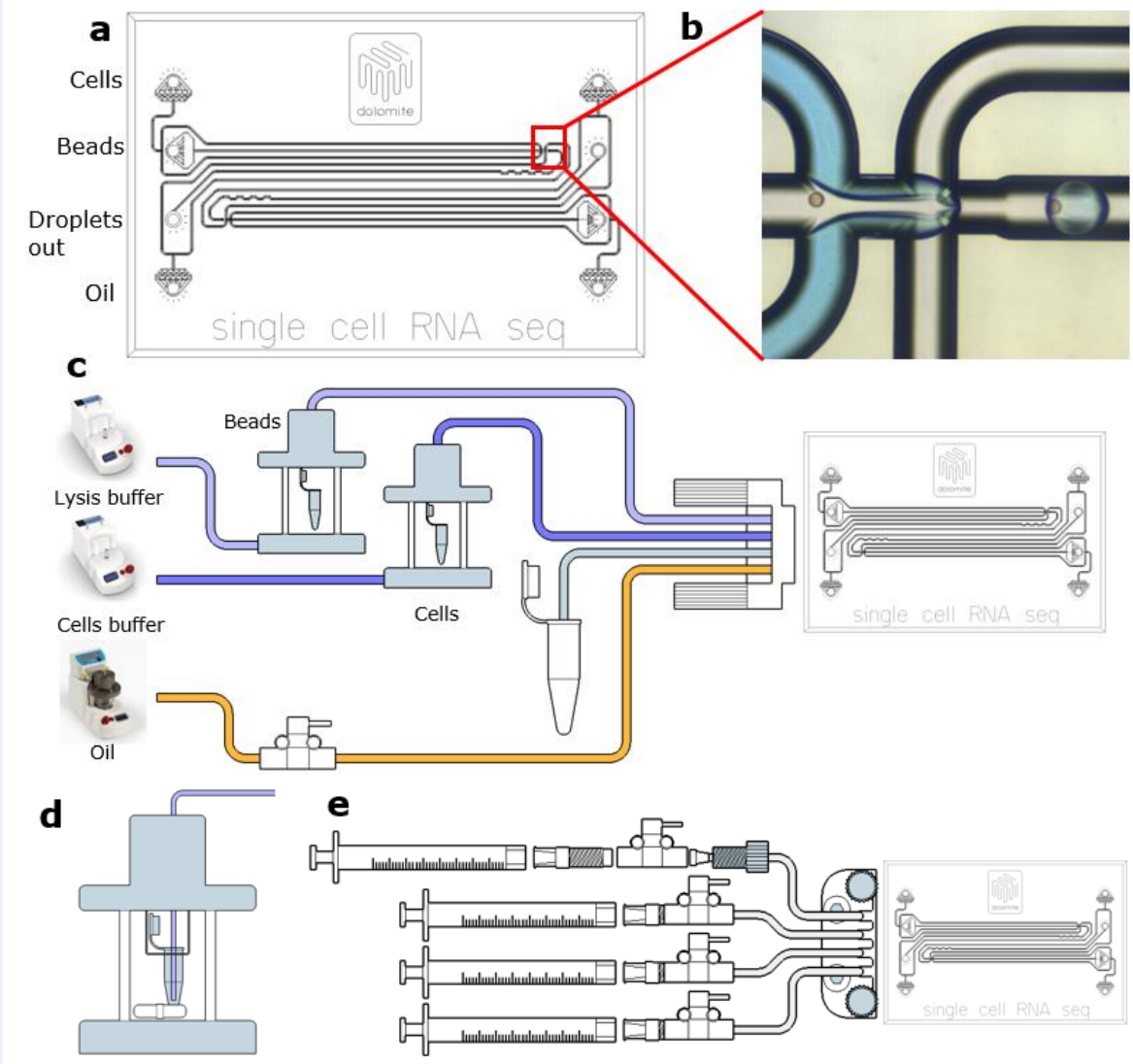


Figure 2. ‘Rotated’ microcentrifuge tubes. a) Drawing of the chip, with two identical circuits. The ports are labelled (“cells”, “Beads”, etc.) for one circuit. b) A photomicrograph of one of the junctions on the chip, making droplets with dye and beads. c) Schematic diagram, showing the system setup. d) Diagram, illustrating how the magnetic stir bar was used to ‘rotate’ a microcentrifuge tube. e) Diagram, illustrating the cleaning station.

via a 16G blunt needle, the needle was removed, the syringe was flicked to dislodge and discard bubbles, and connected to the injection valve via a luer fitting. The beads settle rapidly, so it is useful to gently invert the syringe to keep the beads in suspension. The beads or cells were then injected into the sample loop, taking care to avoid introducing bubbles (which sweep beads along in the sample loop, resulting in a higher local concentration of beads in front of the bubble), or allowing the beads (in particular) to settle during sample injection.

Sample loops on a rocker. These sample loops were 2m of 0.8 mm tubing, with a volume of 1 ml. The sample loops were placed on a rocker that moved 180 degrees, paused, rotated 180 degrees in the reverse direction, paused, etc., so that the beads remained in suspension. In this case, the sample loops were 2m of 1.6 mm OD, 0.8 mm ID, which had a volume of 1 ml. As above, the loops had a 4-way sample injection valve upstream, and were connected downstream via a linear union and 500 mm of 0.25 mm ID tubing to the chip. The whole sample loop, including the injection valve and the downstream union were on the rocker, so that the connections were also rocked.

Other reagents. Cells were suspended in 1X TBS (50 mM TRIS pH 7.4, 150 mM NaCl). Cells were suspended in the buffer, counted in a hemacytometer, and used for a test encapsulation. The cells/droplet were counted, and the cell concentration adjusted as necessary.

Cell & Bead Encapsulation. One P-Pump was loaded with the droplet oil, the 'cells' pump was loaded with the cell suspension buffer, and the 'beads' pump was loaded with lysis buffer. In this case, the buffers loaded into the pumps functioned as 'driving liquids', to push the cell or bead suspensions out of the sample loops. The system was primed by pumping the buffers until drops appeared at the connector, the pumps turned off, and the injection valves turned to the sample injection position. The cell and bead suspensions were loaded into the sample loops. The flows were started essentially as described in Macosko et al., i.e. in the order cells>beads>oil, in order to avoid backflow of lysis solution into the cells tubing line, and premature lysis. Specifically, the pumps were controlled from a program in the Dolomite Flow Control Centre, that rapidly turned on the 'cells' pump to 40 μl/min, then the 'beads' pump to 40 μl/min, then the 'oil' pump to 200 μl/min. This could also be done manually. As described in Macosko et al (Macosko E., et al. "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets." *Cell*

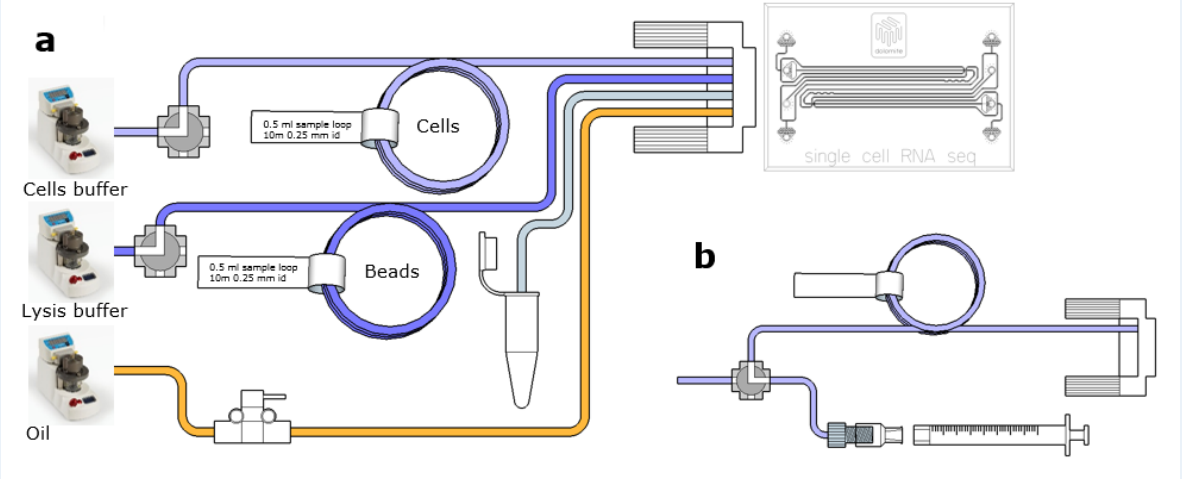


Figure 3. Sample loops. a) Schematic diagram of a system using sample loops with upstream 4-way injection valves. b) The sample loops were loaded with a 1 ml luer lok syringe via a port on the injection valves.

161:1202), the chip does not always initiate droplet formation, and partially or completely 'streams' instead (see Fig. 3b). It is useful if the camera if fast enough to be able to visualise droplets, and therefore confirm that droplet production has correctly initiated. If it is not, gently tapping the upstream tubing (leading from the P-Pumps to the chip) several times generally causes droplet production to initiate. Otherwise, the oil flow should be reduced to 20 μl/min, then increased to 200 μl/min again. Once stable droplet production is initiated (i.e., the droplets are stably forming just after the junction for longer than 5 – 10 seconds), then the outlet tubing should be moved to the collection vessel.

It was possible to run the chip at 67 μl/min (4,000 μl/hour) for each of the aqueous flows, and 250 μl/min (15,000 μl/hour) for the oil, however, at these flow rates, the aqueous flows tend to stream, rather than form droplets from the junction. In this case, the streaming flow would break up into droplets further downstream, and the droplets would be poly-disperse. In general, droplet production was more stable and easily initiated at flow rates of 40 μl/min for each of the aqueous flows, and 200 μl/min for oil. At these flow rates, the droplet formation frequency was slightly over 4,000 droplets per second, almost double the rate of the PDMS chip. If a slower flow rate is desired, the aqueous reagents can be flowed at 30 μl/min each, and the oil at 167 μl/min.

Results

The objectives of testing were 1) to confirm that the chip produces droplets as described for the PDMS prototype, 2) to test that beads and cells were reliably encapsulated, 3) to determine the highest flow rates at which monodisperse droplets could be reliably formed, and 4) to determine the droplet sizes that the chip reliably produces, the appropriate cell and bead suspension concentrations, and the consequent rate of cell encapsulation and single cell library production.

Another consideration is obtaining monodisperse droplets. If the droplets are poly-disperse (i.e., there are larger droplets), then there will be more variance in the rate of cells/droplet, which will result in more doublets, and less clean data.

In order to determine the optimal flow rates, we made droplets at a series of flow rates

Table 2. Experiment 1. Flow and droplet rates for the glass RNA-Seq chip

Aq., X 2, μl/min	30	30	40	40
Oil, μl/min	100	150	150	200
Droplet dia., μm	110	99	92	85
Droplet vol., pl	697	508	408	330
Droplets/second	1,400	1,970	3,300	4,000
Cells/ml, for 1/20 droplets	1.4×10^5	2.0×10^5	2.5×10^5	3.0×10^5

Table 3. Sample loop volume, in μl/meter of tubing

ID, mm	cross section (mm^2)	volume, μl/m of tubing
0.8	0.503	503
0.5	0.196	196
0.25	0.049	49
0.1	0.008	8

(Table 2), with a cell suspension, beads suspended in lysis buffer, and droplet oil, essentially as described (Macosko et al, *Cell* **161**:1202).

Flowing beads. As noted in Macosko et al., flowing beads is the most challenging part of the protocol, as the beads sediment out quite rapidly. In the case of the syringe pumps, the chosen solution was to put a magnetic stirring disc in the syringe, resulting in good suspension of beads, although care had to be taken to avoid shearing beads, leading to less clean data (because fragments of a broken bead, and therefore the same barcode, can be encapsulated with different cells). We briefly investigated different methods of flowing beads using pressure pumps.

One method is to put the sample (of beads or cells) in a microcentrifuge tube in a remote P-Pump chamber, and rotate the microcentrifuge tube by placing a 20 mm stir bar in the chamber and putting the chamber on a stirrer (Figure 2). This is very similar to the method described in the Macosko paper, except the stir bar wasn't immersed in the sample. This resulted in a good flow of beads. The beads were suspended at 3.8 X 10^5 beads/ml. 85 μm droplets have a volume of 330 pl, of which half (165 pl) is bead suspension, so this concentration of beads is equivalent to 6.3% of droplets containing a bead. Droplets were collected and imaged in a hemacytometer. In six fields, 511 beads were counted in 8,300 droplets, i.e., 6.2% of droplets contained a bead. However, some bead damage was observed as also reported by Macosko et al., possibly due to the fragile nature of the beads. The number of damaged beads increased with increase stirring time (not shown), and could be minimised by minimising the stirring time.

We next tested sample loops (Figure 3), with the intention of minimising bead damage. Beads flowed from the sample loops had no noticeable damage. Beads were suspended at 6.2 X 10^5 beads/ml, equivalent to 9.7% of droplets having a bead. 13,300 droplets contained 1,295 beads, for an average of 9.7% of droplets having a bead. However, the

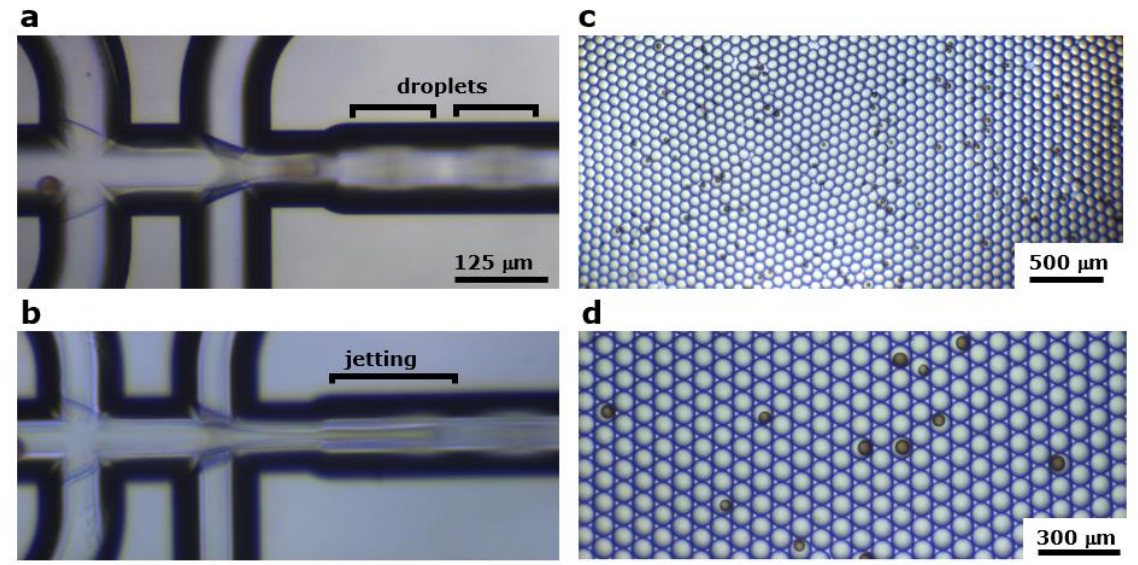


Figure 4. Droplet formation. a) Correct droplet formation just after the junction. Droplets were formed at ~4 kHz (4,000 droplets per second), with flow rates of 40 μl/min for each of the aqueous phases (beads and cells), and 200 μl/min for the oil. The exposure time was 50 μs. Note that even at these high flow rates, droplets are still visible. b) At higher flow rates, the junction tends to 'stream' or 'jet', rather than form droplets, leading to polydisperse emulsions. The flow rates were 50 μl/min for each of the aqueous phases, and 200 μl/min for the oil. c) and d). Emulsions were produced at the optimal flow rates (40, 40 and 200 μl/min) and imaged. Note that the droplets are highly monodisperse.

beads did flow slightly slower than the buffer, which meant it was important to avoid injecting air bubbles into the loops, as air bubbles can sweep beads in front of them, creating a transient high bead concentration. With a low number of cells/droplet, cells were difficult to count confidently, so cells were suspended at $3.7x10^7$ cells/ml, equivalent to 6 cells/droplet, however we only observed 88 cells/55 droplets, or 1.6 cells/droplet, implying that only ~27% of the cells were observed, implying that cells were not swept along as effectively as beads, possibly because the cells are smaller than the beads (we used sheep erythrocytes, which are only ~4.5 μm diameter).

We also placed the sample loops on a rocker, so that the loops could be periodically inverted, to keep beads in suspension. This also allowed us to use 0.8 mm ID tubing, which has an internal volume of 500 μl/m, in contrast to 0.25 mm ID tubing, which only has an internal volume of 50 μl/m. We suspended beads at 3.8 X 10^5 beads/ml. Beads were observed through the wall of the tubing to be in suspension, and to be flowing well. We expected 6.3% of droplets to contain a bead, and counted 707 beads in 12,600 droplets, or 5.6% of droplets containing a bead.

It is generally desired to obtain a high droplet rate, partly to increase possible throughput, and partly to minimise processing time. The highest flow rates that reliably produced mono-disperse droplets were 40 μl/min for each of the two aqueous phases, and 200 μl/min for the oil phase (Figure 4a). The droplets were reliably monodisperse, and contained beads at approximately one bead per 20 droplets (Figure 4c & d; the cells can't be seen, as they have been lysed by the detergent). At higher flow rates (50 μl/min each for the aqueous phases), the junction tended to stream, and produce poly-disperse droplets (Figure 4b). At the flow rates of 40 & 200 μl/min (Figure 3a), the droplets were ~85 μm in diameter, which equates to a volume of 330 pl, and a droplet production rate of 4 kHz (4,000 droplets per second). Interestingly, this is almost twice as fast as that reported for the PDMS prototype chip, resulting in higher throughput.

Based on these preliminary results, the sample introduction for beads and cells can be achieved using the sample loop and a 'stirred' microcentifuge sample tube. However, it is believed that the preferred method is to introduce the cells using a stirred microcentifuge tube and the beads via the sample loop, as shown in Figure 5 below.

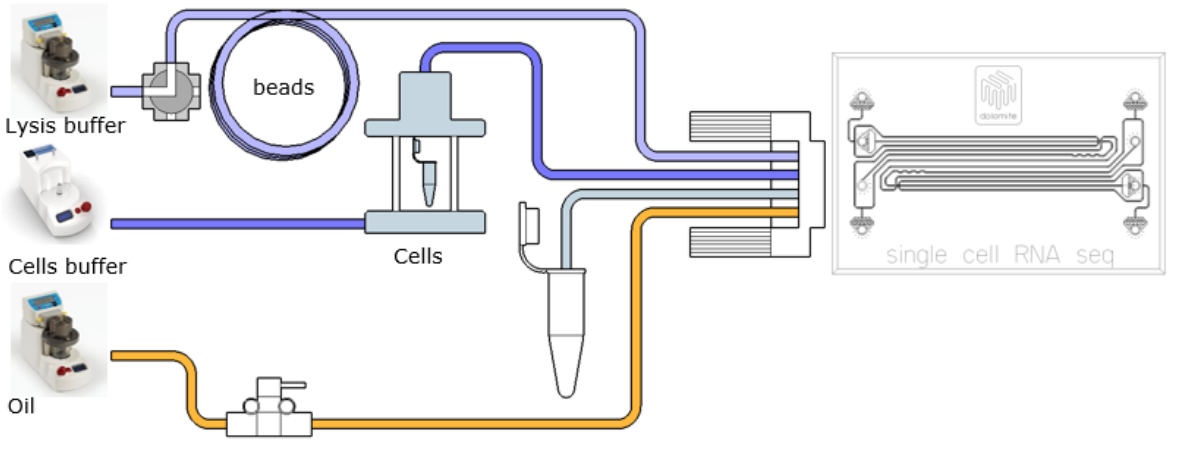


Figure 5. Preferred setup. Cells flow well from a 'stirred' microcentrifuge tube, and beads flow well from a sample loop.

Conclusions

The single cell RNA-Seq protocol offers an extremely powerful and exciting method. This development allows easy, straightforward access to large numbers of single cell expression profiles, for the first time. Consequently, many research groups are eager to adopt the workflow described in the Drop-seq protocol.

It has been shown here that the first generation glass RNA-Seq chip can successfully encapsulate cells with barcoded beads in the lysis buffer, producing monodisperse droplets with highly consistent volumes. Further work can be performed to optimise the method to increase the production rate of single cell libraries. A more rapid production of libraries would minimise incubation time of the cells, and is expected to reduce interference with the expression profiles of cells, producing higher quality data. Further optimisation and development is being carried out within Dolomite to overcome some of the challenges that have been identified within this note.

In conclusion, the RNA-Seq application has been demonstrated with impressive results using a glass chip within a droplet system. The method has proven to be robust in the hands of a skilled researcher. The results presented here show stable droplets, similar to those reported in the original publication, with the advantage of obtaining slightly smaller droplets, resulting in a two-fold increase in droplet production rate.

Appendix A: System Component List

Section	Part No.	Part Description	#
Chip, loops, connectors	3200455	Single Cell RNA-Seq chip 1	2
	3000024	Linear Connector 4-way	1
	3000109	Top Interface 4-way (4mm)	1
	3000477	End Fittings and Ferrules for 1.6mm Tubing (pack of 10)	1
	3200063	FEP Tubing, 1/16" x 0.25mm, 10 meters	3
	3200087	2-way In-line Valve	3
	3000664	1/4 - 28 Modified Luer Fitting	4
	3000311	Female to Female Luer Lock	4
Standard droplet system	3200016	Mitos P-Pump	2
	3200176	Mitos P-Pump Remote	1
	3200178	Mitos P-Pump Remote Chamber 30	1
	3200118 (US) 3200117 3200128 (EU)	Mitos compressor 110V/60Hz Mitos compressor 230V/50Hz Mitos compressor EU 230V/50Hz	1
	2200480	USB hub	1
	3200095	Mitos Sensor Display	3
	3200244	Mitos Flow Rate Sensor, mixed pack of 3	1
	3200017	Mitos P-Pump Vessel Holders Kit	2
Fittings and tubing	3000477	End Fittings and Ferrules for 1.6mm Tubing (pack of 10)	2
	3200060	Flangless Ferrule, ETFE (pack of 10)	1
	3200300	FEP Tubing, 1/16" x 0.1mm, 10 meters	1
	3200063	FEP Tubing, 1/16" x 0.25mm, 10 meters	2
	3200065	FEP Tubing, 1/16” x 0.8mm, 10 meters	1
	3000311	Female to Female Luer Lock	5
	3000664	1/4 - 28 Modified Luer Fitting	5
	3000399	1/4 - 28 Straight Female Coupling, ECTFE	5
	3000397	T-Connector ETFE	2
	C000855	Valve 4-Way PEEK Right Angle Flow	3
Microscope	3200050	High Speed Camera and Microscope System	1

Chip cleaning module	3000024	Linear Connector 4-way	1
	3000109	Top Interface 4-way (4mm)	1
	3000664	1/4 - 28 Modified Luer Fitting	4
	3200087	2-way In-line Valve	4
	3200063	FEP Tubing, 1/16" x 0.25mm, 10 meters	1

www.dolomite-microfluidics.com

dolomite

The Dolomite Centre Ltd (Europe and Rest of World)
T: +44 (0)1763 242 491
E: info@dolomite-microfluidics.com
W: www.dolomite-microfluidics.com

North America - regional office
T: 617 848 1211
E: info@dolomite-microfluidics.com

Japan - regional office
T: 045 263 8211
E: info@dolomite-microfluidics.com

India - regional office
T: +91 22 2686 4410
E: info@dolomite-microfluidics.com

Brasil - regional office
T: +55 11 5083 4963
E: info@dolomite-microfluidics.com